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(56) Documents cited
GB A 2040943 GB 1263408 EP 0061746
GB A 2023607 GB 1090466

(58) Field of search
C2P

(54) Nucleoside phosphoramidites and oligonucleotides produced therefrom

(57) (Protected amino)-derivitised nucleoside phosphoramidies and in particular 5'-deoxy-5'-(protected amino) nucleoside phosphoramidites are used to prepare oligonucleotides. Detectable labels can be attached to the oligonucleotides through the free amino groups on the oligonucleotides which are of use in the detection of genetic diseases by DNA hybridization.

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FIG. 1

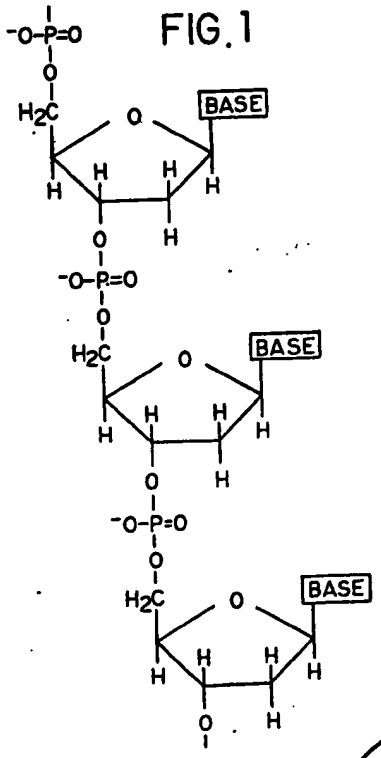


FIG. 2

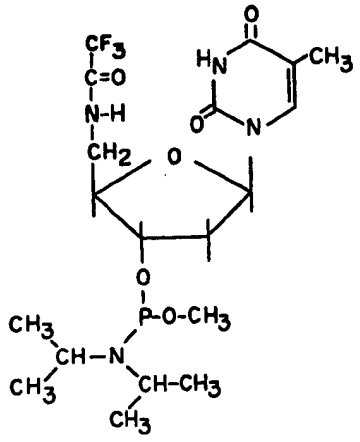
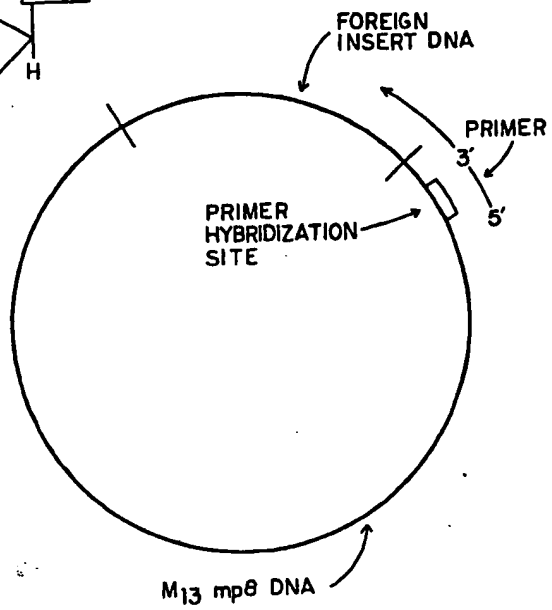


FIG. 4



SINGLE STRANDED RECOMBINANT DNA TEMPLATE FOR DIDEOXY SEQUENCING

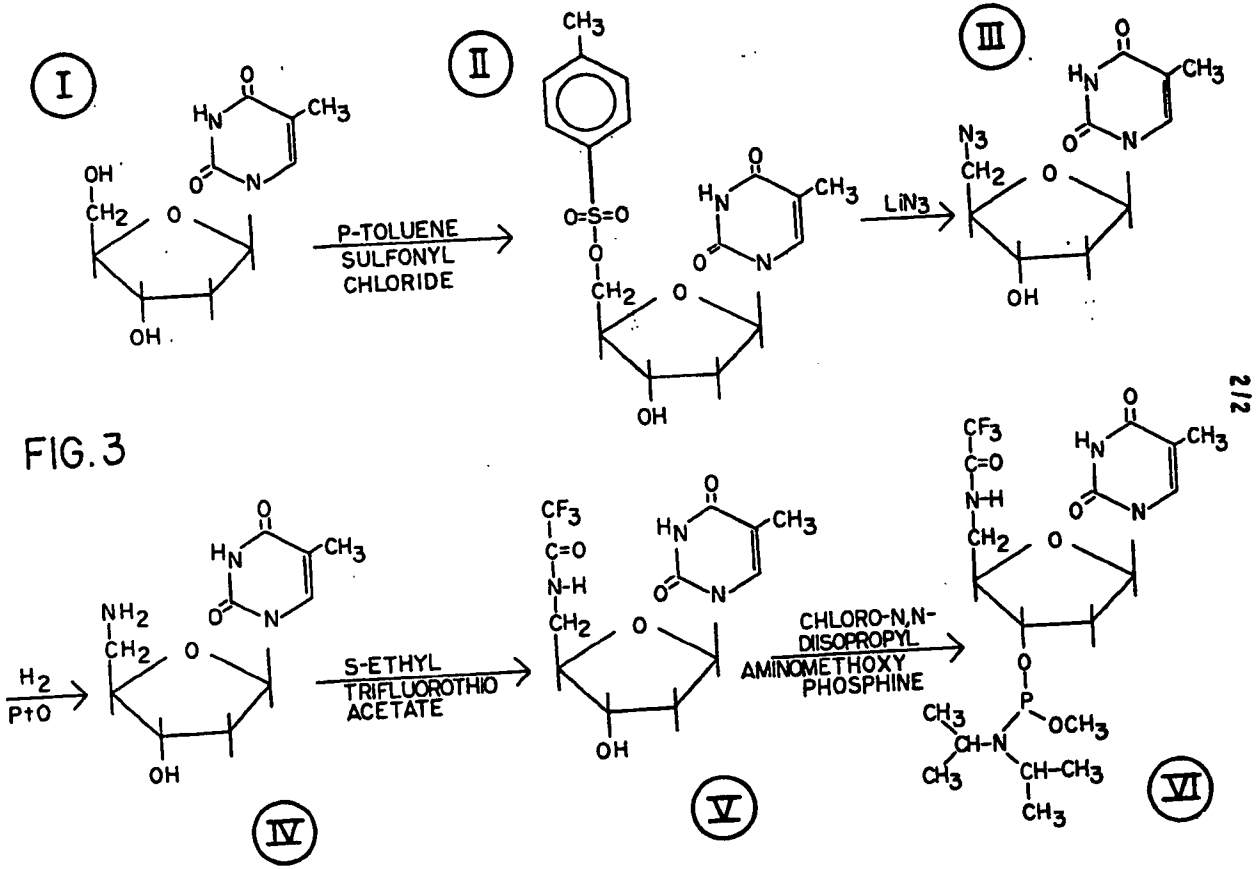


FIG. 3

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SPECIFICATION

Nucleoside phosphoramidites and oligonucleotides produced therefrom

An oligonucleotide is a short polymer consisting of a linear sequence of any of four nucleotides in a defined order. The nucleotide subunits are joined by phosphodiester linkages joining the 3′ hydroxyl moiety of one nucleotide to the 5′ hydroxyl moiety of the next nucleotide. An example of an oligonucleotide is 5′ ApCpGpTpApTpGpGpCp 3′. The letters A, C, G and T refer to the nature of the purine or pyrimidine base coupled at the 1-position of deoxyribose. A, adenine; C, cytosine; G, guanine; T, thymidine. P represents the phosphodiester bond. The structure of a section of an oligonucleotide is shown in Fig. 1.

The single stranded oligonucleotides of this invention are further characterized by being homogenous with respect to the sequence of the nucleoside subunits and are of uniform molecular weight.

Synthetic oligonucleotides are powerful tools in modern molecular biology and recombinant DNA work. There are numerous applications for these molecules, including a) as probes for the isolation of specific genes based on the protein sequence of the gene product, b) to direct the *in vitro* mutagenesis of a desired gene, c) as primers for DNA synthesis on a single-stranded template, d) as steps in the total synthesis of genes, and many more, reviewed in Wm. R. Bahl et al, *Prog. Nucl. Acid Res. Mol. Biol.*, 21, 101 (1978)

A very considerable amount of effort has therefore been devoted to the development of efficient chemical methods for the synthesis of such oligonucleotides. A brief review of these methods as they have been developed to the present is found in Crocket, G. C., *Aldrichimica Acta* 16(3), 47–55 (1983). The best methodology currently available utilizes the phosphoramidite derivatives of the nucleosides in combination with a solid phase synthetic procedure, Matteucci et al, *J. Am. Chem. Soc.*, 103, 3185 (1981); and Beaucage et al, *Tet. Lett.*, 22(20), 1859–1862 (1981). Oligonucleotides of length up to 30 bases may be made on a routine basis in this matter, and molecules as long as 50 bases have been made. Machines that employ this technology are now commercially available.

There are other reports in the literature of the derivitization of DNA. A modified nucleoside triphosphate has been developed wherein a biotin group is conjugated to an aliphatic amino group at the 5 position of uracil, Langer et al, *Proc. Nat. Acad. Sci.*, U.S.A., 78. 6633–6637 (1981). This nucleotide derivative is effectively incorporated into double stranded DNA. Once in DNA it may be bound by anti-biotin antibody which can then be used for detection by fluorescence or enzymatic methods. The DNA which has had biotin conjugated nucleosides incorporated therein by the method of Langer et al is fragmented into smaller single and double stranded pieces which are heterogeneous with respect to the sequence of nucleoside subunits and variable in molecular weight. Draper and Gold, *Biochemistry*, 19, 1774–1781 (1980), reported the introduction of aliphatic amino groups by a bisulfite catalyzed transamination reaction, and their subsequent reaction with a fluorescent tag. In Draper and Gold the amino group is attached directly to a pyridine base. The amino group so positioned inhibits hydrogen bonding and for this reason, these materials are not useful in hybridization and the like. Chu et al, *Nucleic Acid Res.* 11(18), 6513–6529 (1983), have reported a method for attaching an amine to the terminal 5′ phosphate of oligonucleotides or nucleic acids.

There are many reasons to want a method for covalently attaching other chemical species to synthetic oligonucleotides. Flourescent dyes attached to the oligonucleotides permits one to eliminate radioisotopes from the research, diagnostic and clinical procedures in which they are used, and improve shelf-life and availability. As described GB-A-2084899 for a DNA sequencing machine the synthesis of fluorescent-labeled oligonucleotides permits the automation of the DNA sequencing process. The development of appropriate techniques and instrumentation for the detection and use of fluorescent-labeled oligonucleotides allows the automation of other currently laborious laboratory and clinical techniques. The attachment of DNA cleavage chemicals such as those disclosed by Schultz et al, *J. Am. Chem. Soc.*, 104, 6861 (1982); and Hertzberg et al, *J. Am. Chem. Soc.*, 104, 313 (1982) permits the construction of synthetic restriction enzymes, whose specificity is directed by the oligonucleotide sequence.

This present invention presents a general method for the introduction of a free aliphatic amino group(s) into synthetic oligonucleotides. This amino group is readily and specifically reacted with a variety of amino reactive functionalities, and thereby permits the covalent attachment of a wide variety of chemical species.

Briefly, the present invention comprises novel aliphatic aminoderivatized single stranded oligonucleotides conjugated to a detectable moiety which is a chromophore, fluorescent agent, protein, enzyme or other "tag".

This invention further includes a novel oligonucleotides having inserted therein at least one aminoderivitized nucleoside via phosphoramidite precursor.

In another aspect, this invention comprehends the synthesis of oligonucleotides on a

solid phase support, wherein the oligonucleotide is reacted with a protected aminoderivitized nucleoside phosphoramidite.

The invention is in one specific aspect, the synthesized molecule 5'-trifluoroacetamido 5'-deoxy 3'-N,N-diisopropyl phosphoramido thymidine (Fig. 2) and its addition to the 5' terminus as the last addition in solid phase oligonucleotide synthesis. During cleavage and deprotection of the oligonucleotide, the trifluoroacetyl group is hydrolyzed, leaving a free aliphatic amino group on the 5' terminus of the oligonucleotide. This amino-derivitized oligonucleotide may then be reacted with any of a wide variety of amino-reactive molecules to give the corresponding oligonucleotide derivative. This is a special case of the more general approach of using a modified nucleoside which has a protected aliphatic amino group on the base moiety, rather than on the 5' carbon. Such a molecule allows several free amino groups to be placed within the oligonucleotide, and at any desired position in the oligonucleotide sequence.

It is an object of this invention to provide new reagents and techniques applicable to DNA sequencing.

It is also an object of the invention to provide improvements in DNA hybridization for the detection of genetic diseases and for other purposes.

These and other objects and advantages of my invention will be apparent to those skilled in this art from the more specific disclosure which follows.

The strategy used to introduce alphatic amino groups into an oligonucleotide is to synthesize a 3' phosphoramidite derivative of a nucleoside analogue containing a protected aliphatic amino group. This phosphoramidite may then be reacted with the oligonucleotide being synthesized on a solid support in a manner analogous to the reaction of underivitized nucleoside phosphoramidites. Cleavage from the solid phase and deprotection of the base moieties and aliphatic amino group yields the amino-derivitized oligonucleotide.

EXAMPLE I

Synthesis of VI. In Fig. 3, the synthesis of compound VI from the commercially available compound I (thymidine) is shown. The synthesis of compounds II-IV are disclosed in Horowit et al, *J. Org. Chem.*, 27, 3045-3048 (1962); and Gibbs and Orgel, *J. Carbohydrates-Nucleosides and Nucleotides*, 3(5 and 6), 315-334 (1976). The synthesis of compounds V and VI are as follows.

EXAMPLE II

5'trifluoroacetamido 5'deoxythymidine(V): 1.25 gr (5 mmoles) of 5'-amino 5'deoxythymidine was dissolved in 25 ml dry dimethyl formamide. To this was added 1.3 ml (10 mmoles) S-ethyl trifluorothioacetate (Aldrich). The reaction was stirred gently at room temperature. TLC of the reaction mixture on silica gel F-254 plates run in MeOH:Acetone 1:1 show a single spot of product detected by short wave UV. The product has a high mobility in this solvent system in contrast to the starting compound which is virtually immobile.

The reaction mixture was rotary evaporated to dryness under reduced pressure, transferred to an Erlenmeyer flask in 30 ml isopropanol, and recrystallized from boiling Isopropanol:MeOH. Yield = 1.315 gr (3.9 mmoles, 80% yield), m.p. 261°–262° (dec), anal. pred. C, 42.7%; H, 4.18% N, 12.5%, exp. C, 42.7%, H, 4.16%; N, 12.4%. The structure of V was further confirmed by ^{1}H NMR.

EXAMPLE III

This Example illustrates the preparation of a protected aminoderivitized nucleoside phosphoramidite.

5'trifluoroacetamido 5'-deoxy 3'-N,N-diisopropyl phosphoramido thymidine (VI): All glassware, syringes and capillary tubes used in this reaction were baked overnight in a drying oven. Dimethyl formamide (DMF) was stored over 4 Å molecular sieves (Linde). Diisopropyl ethyl amine (DIPEA) was distilled from potassium hydroxide, then from calcium hydride, and stored over 4 Å molecular sieves.

To a dry 3-necked 50 ml round-bottom flask with stir bar was added 63 mg (0.19 mmoles) V. The three necks were plugged with rubber serum stoppers and needles inserted in the stoppers. The flask was pumped on for several hours in a dessicator over dry $CaCl_2$. The flask was kept under a gentle stream of dry nitrogen gas and 2 ml of dry DMF added by syringe. 60μl of DIPEA (0.34 mmoles) was added in a dry 100 μl capillary tube. 40 μl of chloro-N,N-diisopropyl amino methoxy phosphine (American Bionuclear, Emeryville, CA) was added (in a dry 100 μl capillary tube). The reaction was stirred gently until all starting material was dissolved, and then allowed to sit at room temperature (always under N_2). TLC after one hour on silica gel F-254 plates in $HCCl_3$:EtOH:Et_3N 88:10:2 showed a single spot of product of much higher mobility than the starting material V. Attempts to purify this product in a fashion similar to that described for the nucleoside phosphoramidites (4) were unsuccessful due to degradation of the product. Therefore, the crude reaction mixture was used directly for the coupling to the synthetic oligonucleotide on the solid phase support. The structure of VI is inferred from the reactivity of this product in the addition to the oligonucleotide, and from the expected product of the reaction based on literature results.

EXAMPLE IV

This Example illustrated the preparation of

an oligonucleotide coupled at the 5′ terminus via a phosphodiester linkage to the 3′ hydroxyl of 5′-amino 5′-deoxythymidine.

Addition of VI to the 5′ terminus of an oligonucleotide: A base-protected synthetic oligonucleotide of sequence 5′OH-AGC ACT TTT AGA GT 3′ coupled to the solid phase at the 3′ terminus was prepared by methods which are described in detail in the protocol entitled "A Procedure for the Manual Synthesis of Deoxyoligonucleotides using dimethoxytrityl nucleoside phosphoramidites on a Solid Support", issued by Applied Biosystems, Inc., Foster City, California. The differences for reaction of the oligonucleotide with VI are a) in place of steps 4.22 and 4.23, 1 ml of the freshly prepared reaction mixture containing VI is combined with 1 ml of 0.5M tetrazole in acetonitrile and added to the reaction vessel; b) after step 4.33 there are two 30-second washes with acetonitrile; and c) the capping section 5 is omitted (so as to allow for assay of unreacted hydroxyl groups in a subsequent addition).

The efficiency of the coupling reaction is readily monitored by subsequent coupling with a "normal" phosphoramidite and cleavage of the dimethoxy trityl group to give a color assay. If the 5′ hydroxyl groups of the oligonucleotide reacted in the first coupling with VI, they are no longer available for reaction in the subsequent coupling, and there will be little color released upon DCA treatment following the second coupling. In this example $OD_{450} = 1.12$ (after dilution) for the DMT group released from the oligonucleotide prior to reaction with VI. $OD_{450} = 0.026$ (after dilution) for the DMT released from a G residue added subsequent to reaction with VI. Therefore 98% of the 5′ OH groups were blocked by reaction with VI. The oligonucleotide was deprotected and cleaved from the solid phase by treatment with thiophenol and concentrated NH_4OH in the usual manner, and the NH_4OH removed under reduced pressure. The oligonucleotide was dissolved in 1 ml of distilled H_2O. The OD_{260} was 128 of this solution, indicating the concentration of DNA to be 4.5 mg/ml. 50μl of this solution was diluted to 1 ml with H_2O and mixed for 15 min with a few hundred mg of AG50W-X4 (sodium form) ion exchange resin to convert the DNA from the ammonium salt to the sodium salt (the ammonium ions interfere with the subsequent ninhydrin assay). The resin was removed by centrifugation, and the supernatant dried down in a Savant rotary concentrator. Quantitative ninhydrin assay (Sarin, V. K., et al, *Anal. Biochem. 117*, 147-157 (1981)), gave approximately 1 mole of amino group per mole oligonucleotide (the molar concentration of oligonucleotide was determined from a calculated extinction coefficient $E_{260} = 1.55 \times 10^5$, based on nucleotide composition). Ninhydrin assay on the same molar amount of a control oligonucleotide to which no VI had been conjugated gave no amino positive-reaction.

EXAMPLE V

Conjugation with dye: To 100 μl of the above solution of amino oligonucleotide was added 200 μl H_2O, 50 μl of 1 M carbonate/bicarbonate buffer pH 9.0, and 25 μl of freshly prepared 10 mg/ml fluorescein isothiocyanate (FITC) (Molecular Probes, Inc., Junction City, Oregon) in dimethyl sulphoxide. The mixture was left at room temperature for several hours, and purified by chromatography on a column (1cm × 9cm) of Sephadex G-25 medium in H_2O. The yellow product eluted in the excluded volume and was cleanly resolved from unreacted dye. A control reaction with oligonucleotide to which no VI had been reacted gave very little or no color in the excluded volume of the column, indicating that the dye was indeed reacting with the added amino group. The dye-oligonucleotide conjugate had $OD_{260} = 2.3$, $OD_{495} = 0.54$. Based on E_{495} 7×10^4 for FITC, this gives a 7.7 μM solution of dye. Based on $E_{260} = 1.55 \times 10^5$, the DNA is 12.8 μM. Therefore ~60% of the DNA molecules were labeled with dye. This is a rough estimate, which does not allow for changes in the bound fluorescein absorption relative to unbound, nor for contaminating shorter and non-reactive oligonucleotides. An aliquot of this colored DNA was electrophoresed on a 20% polyacrylamide gel and was clearly visible as a single colored (and fluorescent) band of a mobility appropriate for an oligonucleotide of that length.

The dye conjugated oligonucleotide was readily purified by high performance liquid chromatography (HPLC) on a reverse phase C_{18} column (Waters) using an acetonitrile:0.1 M triethyl ammonium acetate pH 7.0 gradient for elution.

There are numerous possible applications of the novel amino-derivitized oligonucleotides which have been disclosed above. The aliphatic amino group is easily and specifically reacted with a large number of functional groups. This means that virtually any desired molecule may be attached to an oligonucleotide prepared as described above. This includes enzymes, other proteins, fluorescent tags, bioluminescent tags, chromophores, and so on. Oligonucleotides have been widely used in a number of areas, often in conjunction with radiolabels. It will be possible to substitute non-radioactive probe molecules for the radioactive labels. This will make procedures utilizing oligonucleotides cheaper and easier to use, and compatible with a clinical setting. While radiolabels are less preferred, the novel amino-derivatized oligonucleotides can also be radiolabeled, for example, such as with I^{125}. The following three particular

examples of uses of the novel amino-derivitized oligonucleotides are illustrative only:

1. Automated DNA sequencing.

2. Detection of genetic disease by DNA hybridization.

3. General use of fluorescence for detection of hybridization.

Detection of genetics Abnormalities: Oligonucleotides may be used to determine the genotype of individuals. This is done on DNA samples obtained from the fetus by aminiocentesis. This information is invaluable for the genetic counseling of couples at risk for a variety of genetic diseases. The genotype of adults may also be determined, allowing effective diagnosis and treatment at an early stage. One striking example of this technology is for the detection of sickle cell anemia, Connor et al., *Proc. Natl. Acad. Sci.* USA 80, 278 (1983). Nineteen-base-pair-long synthetic oligonucleotides were synthesized, one complementary to the normal human β-globin gene (β^A), and one complementary to the sickle cell β-globin gene (β^S). These molecules were radioactively labeled and used as probes in DNA hybridization. Under appropriate hybridization conditions, these probes can be used to distinguish the β^A gene from the β^S allele. This allows diagnosis of the sickle cell disease. More generally, as pointed out in Connor et al, "this allele-specific hybridization behavior of oligonucleotides provides a general method for the diagnosos of any genetic disease which involves a point mutation in the DNA sequence of a single-copy gene." The present invention is directly applicable to this technique. The oligonucleotide probes are prepared with an amino group and labeled with a fluorescent tag. The fluorescent probe molecule is stable indefinitely, in contrast to the short lifetime of radioactive probes and requires no special precautions for use or handling. This makes such an approach vastly preferable for use in a clinical setting, which is the major area in which the technology will be used.

Oligonucleotide probes are widely used in research work as well as clinical work. They are commonly used to detect a piece of DNA and a desired sequence in a "library," a collection of DNA fragments cloned into a plasmid or phage vector, which contains sequences encompassing the entire genome (or expressed RNA) of an organism. They are also used to hybridize to DNA of a given sequence in a "blot" of a restriction digest of a particular piece of DNA. In all these examples, as well as others, the oligonucleotide is labeled with 32P, usually at the 5′ terminus, and the molecules are detected by autoradiography. The present invention describes the labeling of oligonucleotides with fluorescent dyes. Therefore, fluorescence may be used for detection of the molecules in any of the techniques in which radioactivity has been conventionally used. This present numerous advantages over radioactivity such as stability of the probes, expense and ease of use and disposal.

CLAIMS

1. A (protected amino)-derivitised nucleoside phosphoramidite.

2. A 5′-deoxy-5′-(protected amino) nucleoside phosphoramidite.

3. A compound according to claim 2, in which the protected amino group is a trifluoroacetamido group.

4. A compound according to claim 1, in which the base moiety carries the protected amino group.

5. A compound according to any one of the preceding claims, which is a 2′-deoxy compound.

6. 5′-Deoxy-5′-trifluoracetamido-3′-N,N-diisopropyl-phosphoramido thymidine.

7. A process for the preparation of a (protected amino)-derivitised nucleoside phosphoramidite, which process comprises synthesizing a 3′-phosphoramidite derivative of a nucleoside analogue containing a protected amino group.

8. A process for the preparation of a 5′-deoxy-5′-trifluoroacetamido-thymidine phosphoramidite, which process comprises reacting 5′-deoxy-5′-trifluoroacetamido-thymidine with a phosphine.

9. A process according to claim 8 substantially as hereinbefore described in Example III.

10. An oligonucleotide which has had inserted therein at least one amino-derivitised nucleoside via a phosphoramidite precursor.

11. An oligonucleotide in which the 5′-terminal nucleotide structural unit has a 5′-deoxy-5′-amino terminus.

12. An oligonucleotide according to claim 11, in which the 5′-terminal nucleotide structural unit is a 2′-deoxy nucleotide unit.

13. An oligonucleotide according to claim 11 or 12, in which the 5′-terminal nucleotide structural unit is 5′-deoxy-5′-amino-thymidine.

14. An oligonucleotide according to claim 11 substantially as hereinbefore described in Example IV.

15. A process for the preparation of an oligonucleotide as claimed in claim 10, which process comprises reacting an oligonucleotide attached to a solid phase support with a (protected amino)-derivitised nucleoside phosphoramidite.

16. A process for the preparation of an oligonucleotide as claimed in claim 11, which process comprises reacting an oligonucleotide attached to a solid phase support with a 5′-deoxy-5′-(protected amino) nucleoside phosphoramidite.

17. A process according to claim 16, in which the protected amino group is a trifluoroacetamido group.

18. A process according to claim 16 or 17, in which the nucleoside phosphoramidite is a 2′-deoxy compound.

19. A process according to claim 18, in which the 2′-deoxy compound is a 5′-deoxy-5′-trifluoroacetamido-thymidine phosphoramidite.

20. A process according to claim 19, in which the 2′-deoxy compound is 5′-deoxy-5′-trifluoroacetamido-3′-N,N-diisopropylphosphoramido thymidine.

21. A process according to claim 16 substantially as hereinbefore described in Example IV.

22. A conjugate of an oligonucleotide as claimed in any one of claims 10 to 14 or which has been prepared by a process as claimed in any of claims 15 to 21 and a detectable moiety, the detectable moiety being linked to the oligonucleotide through the free amino group on the nucleotide.

23. A conjugate according to claim 22, in which the detectable moiety is fluorescent.

24. A conjugate according to claim 22, in which the detectable moiety is absorptive or colored.

25. A conjugate according to claim 22, in which the detectable moiety is a protein.

26. A conjugate according to claim 22, in which the detectable moiety is an enzyme.

27. A conjugate according to claim 22, in which the detectable moiety is radioactive I^{125}.

28. A conjugate substantially as hereinbefore described in Example V.

29. The composition comprising aliphatic amino-derivitised single stranded oligonucleotides conjugated to a detectable moiety.

30. The composition of claim 29 wherein the detectable moiety is fluorescent.

31. The composition of claim 29 wherein the detectable moiety is absorptive or colored.

32. The composition of claim 29 wherein the detectable moiety is a protein.

33. The composition of claim 29 wherein the detectable moiety is an enzyme.

34. The composition of claim 29 wherein the detectable moiety is radioactive I^{125}.

35. As a new composition(s) of matter, an oligonucleotide which has had inserted at least one amino-derivitized nucleoside via phosphoramidite precursor(s).

36. In the synthesis of oligonucleotides on a solid phase support, the improvement wherein the oligonucleotide is reacted with a protected amino-derivitized nucleoside phosphoramidite.

37. As a new composition of matter an oligonucleotide coupled at the 5′-terminus via a phosphodiester linkage to the 3′-hydroxy of 5′-amino 5′-deoxythymidine.

38. The method of preparing the composition of claim 37 which comprises reacting 5′-amino 5′-deoxythymidine with a protecting agent to yield 5′ trifluoroacetamido 5′-deoxythymidine which is then reacted with a phosphine to yield the corresponding phosphoramidite, and wherein the phosphoramidite is subsequently reacted with a base protected oligonucleotide attached to a solid phase support.

39. The phosphoramidite obtained by reacting 5′-trifluoroacetamido 5′-deoxythymidine with a phosphine.

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